OK, enter the amendment of specification only
Buoguen Li
10/03/2005

Amendments to the Specification:

The paragraph on page 17, line 17, has been amended as follows:

As used herein, the term "non-toxin protein" or "non-toxin protein sequence" refers to that portion of a fusion protein which comprises a protein or protein sequence which is not derived from a bacterial toxin protein.

The paragraph on page 17, line 20, has been amended as follows:

The term "protein of interest", as used herein, refers to the protein whose expression is desired within the fusion protein. In a fusion protein the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

The paragraph on page 18, line 22, has been amended as follows:

The term "recombinant DNA molecule", as used herein, refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The paragraph on page 18, line 25, has been amended as follows:

The term "recombinant protein" or "recombinant polypeptide", as used herein, refers to a protein molecule which is expressed from a recombinant DNA molecule.

The paragraph on page 19, line 1, has been amended as follows:

The term "native protein", as used herein, refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

The paragraph on page 19, line 3, has been amended as follows:

As used herein, the term "portion" when used in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

The paragraph on page 19, line 7, has been amended as follows:

As used herein, the term "soluble" when used in reference to a protein produced by recombinant DNA technology in a host cell is a protein which exists in solution in the cytoplasm of the host cell; if the protein contains a signal sequence the soluble protein is exported to the periplasmic space in bacteria hosts and is secreted into the culture medium in eucaryotic cells capable of secretion or by bacterial host possessing the appropriate genes (*i.e.*, the *kil* gene). In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called inclusion bodies) in the host cell. High level

expression (*i.e.*, greater than 10-20 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

The paragraph on page 21, line 1, has been amended as follows:

The term "pyrogen", as used herein, refers to a fever-producing substance. Pyrogens may be endogenous to the host (*e.g.*, prostaglandins) or may be exogenous compounds (*e.g.*, bacterial endo- and exotoxins, nonbacterial compounds such as antigens and certain steroid compounds, etc.). The presence of pyrogen in a pharmaceutical solution may be detected using the U.S. Pharmacopeia (USP) rabbit fever test (United States Pharmacopeia, Vol. XXII (1990) United States Pharmacopeial Convention, Rockville, MD, p. 151).

The paragraph on page 21, line 8, has been amended as follows:

The term "endotoxin", as used herein, refers to the high molecular weight complexes associated with the outer membrane of gram-negative bacteria. Unpurified endotoxin contains lipids, proteins and carbohydrates. Highly purified endotoxin does not contain protein and is referred to as lipopolysaccharide (LPS). Because unpurified endotoxin is of concern in the production of pharmaceutical compounds (*e.g.*, proteins produced in *E. coli* using recombinant DNA technology), the term endotoxin as used

herein refers to unpurified endotoxin. Bacterial endotoxin is a well known pyrogen.

The paragraph beginning on page 21, line 15, has been amended as follows:

As used herein, the term "endotoxin-free" when used in reference to a composition to be administered parenterally (with the exception of intrathecal administration) to a host means that the dose to be delivered contains less than 5 EU/kg body weight [FDA Guidelines for Parenteral Drugs (December 1987)]. Assuming a weight of 70 kg for an adult human, the dose must contain less than 350 EU to meet FDA Guidelines for parenteral administration. Endotoxin levels are measured herein using the Limulus Amebocyte Lysate (LAL) test (Limulus Amebocyte Lysate Pyrochrome™, Associates of Cape Cod, Inc. Woods Hole, MA). To measure endotoxin levels in preparations of recombinant proteins, 0.5 ml of a solution comprising 0.5 mg of purified recombinant protein in 50 mM $NaPO_4$, pH 7.0, 0.3M NaCl and 10% glycerol is used in the LAL assay according to the manufacturer's instructions for the endpoint chromogenic without diazo-coupling method [the specific components of the buffer containing recombinant protein to be analyzed in the LAL test are not important; any buffer having a neutral pH may be employed (see for example, alternative buffers employed in Examples 34, 40 and 45)]. Compositions containing less than or equal to [[than]] 250 endotoxin units (EU)/mg of purified recombinant protein are herein defined as "substantially endotoxin-free." Preferably the composition contains less than or equal to 100, and most preferably less than or equal to 60, (EU)/mg of purified recombinant protein. Typically, administration of bacterial toxins or toxoids to adult humans for the purpose of vaccination involves doses of about 10-500 μg protein/dose. Therefore, administration of 10-500 μg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 60 EU/mg

protein, results in the introduction of only 0.6 to 30 EU (*i.e.*, 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose). Administration of 10-500 μg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 250 EU/mg protein, results in the introduction of only 2.5 to 125 EU (*i.e.*, 0.7 to 36% of the maximum allowable endotoxin burden per parenteral dose).

The paragraph on page 23, line 8, has been amended as follows:

As used herein, the term "immunogenically-effective amount" refers to that amount of an immunogen required to invoke the production of protective levels of antibodies in a host upon vaccination.

The paragraph on page 23, line 15, has been amended as follows:

As used herein, the terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

The paragraph beginning on page 29, line 21, has been amended as follows:

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against *Clostridium* species, their toxins, enzymes or other metabolic by-products, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these

antibodies will be produced by immunization of humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *Clostridium* species are contemplated as immunogens. Examples of these toxins include the neuraminidase toxin of *C. butyricum*, *C. sordellii* toxins HT and LT, toxins A, B, C, D, E, F, and G of *C. botulinum* and the numerous *C. perfringens* toxins. In one preferred embodiment, toxins A, B, and E of *C. botulinum* are contemplated as immunogens. Table 2 [[above]] below lists various *Clostridium* species, their toxins and some antigens associated with disease.

The paragraph on page 90, line 12, has been amended as follows:

Protein expression from the three expression constructs made in (a) was induced, and analyzed by Western blot analysis with an affinity purified, goat polyclonal antiserum directed against the toxin A toxoid (Tech Lab). The procedures utilized for protein induction, SDS-PAGE, and Western blot analysis are described in detail in Williams *et al* (1995), *supra*. In brief, 5 ml 2X YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter, pH 7.5+100 μg/ml ampicillin were added to cultures of bacteria (BL21 for pMA1 and pGEX plasmids, and BL21(DE3)LysS for pET plasmids) containing the appropriate recombinant clone which were induced to express recombinant protein by addition of IPTG to 1 mM. Cultures were grown at 37°C, and induced when the cell density reached 0.5 OD_{600}. Induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in a microfuge), and resuspension of the pelleted bacteria in 150 μl of 2x SDS-PAGE sample buffer [Williams *et al.* (1995), *supra*]. The samples were heated to 95°C for 5 min, [[the]] then cooled and 5

or 10 µl aliquots loaded on 7.5% SDS-PAGE gels. BioRad high molecular weight protein markers were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. Western blots, (performed as described in Example 3) which detect toxin A reactive protein in cell lysates of induced protein from the three expression constructs are shown in Figure 7. In this figure, lanes 1-3 contain cell lysates prepared from *E. coli* strains containing pPA1100-2860 in Bl21(DE3)lysE cells; lanes 4-6 contain cell lysates prepared from *E. coli* strains containing pPA1100-2860 in Bl21(DE3)lysS cells; lanes 7-9 contain cell lysates prepared from *E. coli* strains containing pMA30-660; lanes 10-12 contain cell lysates prepared from *E. coli* strains containing pMA660-1100. The lanes were probed with an affinity purified goat antitoxin A polyclonal antibody (Tech Lab). Control lysates from uninduced cells (lanes 1, 7, and 10) contain very little immunoreactive material compared to the induced samples in the remaining lanes. The highest molecular weight band observed for each clone is consistent with the predicted size of the full length fusion protein.

The paragraph on page 122, line 11, has been amended as follows:

Collectively, these results demonstrate that expression of the toxin A repeat region in *E. coli* results in the production of insoluble recombinant protein, when expressed as either large (aa 1870-2680) or small (aa 1870-2190 or aa 2250-2680) fragments, in a variety of expression vectors (native or ~~poly-his~~ poly-His tagged pET, pGEX or PinPoint™-Xa vectors), utilizing growth conditions shown to enhance protein solubility. The exception to

this rule were fusions with the MBP, which enhanced protein solubility, either partially (pMA1870-2190) or fully (pMA1870-2680).

The paragraph on page 126, line 1, has been amended as follows:

The inclusion body preparation was used to immunize hens. The protein was dialyzed into PBS and emulsified with approximately equal volumes of CFA for the initial immunization or IFA for subsequent booster immunizations. On day zero, for each of the recombinant ~~reecombinant~~ preparations, two egg laying white Leghorn hens were each injected at multiple sites (IM and SC) with 1 ml of recombinant protein-adjuvant mixture containing approximately 0.5-1.5 mg of recombinant protein. Booster immunizations of 1.0 mg were given [[of]] on days 14 and day 28. Eggs were collected on day 32 and the antibody isolated using PEG as described in Example 14(a). High titers of toxin A specific antibodies were present (as assayed by ELISA, using the method described in Example 13). Titers were determined for both antibodies against recombinant polypeptides pPA1870-2680 and pMA1870-2680 and were found to be comparable at >1:62,500.

The paragraph on page 191, line 19, has been amended as follows:

A soluble lysate (in Novagen IX binding buffer) from an induced 1 liter 2X YT culture was prepared as described above. Briefly, the culture of pHisBot [Bl21(DE3)pLysS host] was grown at 37°C to an OD_{600} of 0.7 in 1 liter of 2X YT medium containing 100 μg/ml ampicillin, 34 μg/ml chloramphenicol and 0.2% glucose. Protein expression was induced by the addition of IPTG to 1 mM. Three hours after the addition of the IPTG, the cells were cooled for 15 min in [[a]] an ice water bath and then centrifuged 10 min

at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The pellets were resuspended in a total volume of 40 mls Novagen 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), transferred to two 35 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were thawed and the cells were lysed by sonication (4 X 20 second bursts using a Branson Sonifier 450 with a power setting of 6-7) on ice. The suspension was clarified by centrifugation for 20 min at 9,000 rpm (10,000 x *g*) in a JA-17 rotor (Beckman).

The paragraph beginning on page 195, line 29, has been amended as follows:

All cultures were grown in 15 ml tubes (Falcon #2057). All culture medium was prewarmed overnight at the appropriate temperature and were supplemented with 100 μg/ml ampicillin and 0.2% glucose. Terrific broth contains 12 g/l bacto-tryptone, 24 g/l bacto-yeast extract and 100 ml/l of a solution comprising 0.17 M KH_2PO_4, 0.72 M K_2HPO_4. Cultures were grown in [[a]] an incubator on a rotating wheel (to ensure aeration) to an OD_{600} of approximately 0.4, and induced by the addition of IPTG. In all cases, high level expression of insoluble pHisBot protein was observed, regardless of temperature, medium or inducer concentration.

The paragraph on page 196, line 7, has been amended as follows:

The effect of varying the concentration of IPTG upon 2X YT cultures grown at 23°C was then investigated. IPTG was added to a final concentration of either 1 mM, 0.1 mM, 0.05 mM or 0.01 mM. At this temperature, similar levels of ~~pHis-Bot~~ pHisBot protein was induced in the presence of either 1 or 0.1 mM IPTG; these levels of expression was lower than that observed at higher

temperatures. Induced protein levels were reduced at 0.05 mM IPTG and absent at 0.01 mM IPTG (relative to 1.0 and 0.1 mM IPTG inductions at 23°C). However, no conditions were observed in which the induced pHisBot protein was soluble in this host. Thus, although expression levels are superior in the BL21(DE3) host (as compared to the BL21(DE3)pLysS host), conditions that facilitate the production of soluble protein in this host could not be identified.

The paragraph on page 200, line 7, has been amended as follows:

It was determined that quantitative binding of pHisBot protein using the batch format occurred after 3 hrs (Figure 30), but not after 1 hr of binding at 4°C (the resin was stirred during binding). Figure 30 depicts a ~~Coomaisse~~ Coomassie blue stained SDS-PAGE gel (7.5% acrylamide) containing samples of proteins isolated during the purification of pHisBot protein from lysate prepared from the BL21(DE3)pLysS host. Each lane was loaded with 5 µl of protein sample mixed with 5 µl of 2X sample buffer and processed as described in Example 22b. Lane 1 contains high molecular weight protein markers (BioRad). Lanes 2 and 3 contain protein eluted from the Ni-NTA resin. Lane 4 contains soluble protein after a 3 hr batch incubation with the Ni-NTA resin. Lanes 5 and 6 contain soluble and total protein, respectively. Figure 30 demonstrates that the pHisBot protein is completely soluble [compare Lanes 5 and 6 which show that a similar amount of the 50 kD pHisBot protein is seen in both; if a substantial amount (greater than 20%) of the pHisBot protein were partially insoluble in the host cell, more pHisBot protein would be seen in lane 6 (total protein) as compared to lane 5 (soluble protein)]. Figure 30 also demonstrates that the pHisBot protein is completely removed from the lysate after batch absorption with the Ni-NTA resin for 3 hours (compare Lanes 4 and 5).

The paragraph on page 205, line 7, has been amended as follows:

In Example 22 above, a synthetic gene was used to express the C fragment of *C. botulinum* serotype A toxin in *E. coli*. The synthetic gene replaced non-preferred (*i.e.*, rare) codons present in the C fragment gene with codons which are preferred by *E. coli*. The synthetic gene was generated because it [[was]] has been reported that genes which have a high A/T content (such as most clostridial genes) creates expression difficulties in *E. coli* and yeast. Furthermore, LaPenotiere *et al.* suggested that problems encountered with the stability (non-fusion constructs) and solubility (MBP fusion constructs) of the C fragment of *C. botulinum* serotype A toxin when expressed in *E. coli* was most likely due to the extreme A/T richness of the native *C. botulinum* serotype A toxin gene sequences (LaPenotiere, *et al, supra*).

The paragraph beginning on page 206, line 28, has been amended as follows:

An expression vector containing the native *C. botulinum* serotype A C fragment gene was created by ligation of the *NcoI-HindIII* fragment containing the C fragment gene from the pCRScript clone to *NheI-HindIII* restricted pETHisa vector (Example 18b). The *NcoI* and *NheI* sites were filled in using the Klenow enzyme prior to ligation; these sites were thus blunt-end ligated together. The resulting construct was termed pHisBotA (native). pHisBotA (native) expresses the *C. botulinum* serotype A C fragment with a his-tagged N terminal extension which has the following sequence: MetGlyHisHisHisHisHisHisHisHisHisSerSer

GlyHis*IleGluGlyArg*HisMetAla (SEQ ID NO:24), where the underlining represents amino acids encoded by the C. *botulinum* C fragment gene (this N terminal extension contains the recognition site for Factor Xa protease, shown in italics, which can be employed to ~~removed~~ remove the polyhistdine tract from the N-terminus of the fusion protein). The pHisBot (native) construct expresses the identical protein as the pHisBot construct (Ex. 24c; herein after the pHisBotA) which contains the synthetic gene.

The paragraph on page 208, line 7, has been amended as follows:

The cells were cooled for 15 min in [[a]] an ice water bath and then centrifuged for 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The cell pellets were resuspended in a total volume of 40 mls 1X binding buffer (40 mM imidazole, 0.5 M NaCl, 50 mM $NaPO_4$, pH 8.0), transferred to two 50 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were then thawed and the cells were lysed by sonication (using four successive 20 second bursts) on ice. The suspension was clarified by centrifugation 20-30 min at 9,000 rpm (10,000*g*) in a JA-17 rotor. The soluble lysate was batch absorbed to 7 ml of a 1:1 slurry of NiNTA resin:binding buffer by stirring 2-4 hr at 4°C. The slurry was centrifuged for 1 min at 500*g* in 50 ml tube (Falcon), resuspended in 5 mls binding buffer and poured into a 2.5 cm diameter column (BioRad). The column was attached to a UV monitor (ISCO) and the column was washed with binding buffer until a baseline was established Imidazole was removed by washing with 50mM $NaPO_4$, 0.3 M NaCl, 10% glycerol, pH 7.0 and bound protein was eluted using 50mM $NaPO_4$, 0.3 M NaCl, 10% glycerol, pH 3.5-4.0.

The paragraph on page 210, line 14, has been amended as follows:

The p6HisBotA(syn) construct was generated as described below; the term "syn" designates the presence of synthetic gene sequences. This construct expresses the C ~~fragment~~ fragment of the *C. botulinum* serotype A toxin with a histidine-tagged N terminal extension having the following sequence: MetHisHisHisHisHisHisMetAla (SEQ ID NO:32); the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type.

The paragraph on page 224, line 16, has been amended as follows:

A PEI-clarified lysate (0.2% final ~~concentration~~ concentration PEI) [850 ml from 130 gm cell pellet (2 liters fermentation harvest)] was purified on a large scale IDA column. A total of 78 mg of protein was eluted. Extracts from the purification were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. The elution was found to contain an approximately 1:1 mix of BotA/chaperone protein (Figure 32). PEI lysates prepared in this manner were typically 16 OD_{280}/ml. This was estimated to be 8 mg protein/ml of lysate (by BCA assay). Thus, the eluted recombinant BotA protein represented 0.55% of the total soluble cellular protein applied to the column.

The paragraph on page 227, line 14, has been amended as follows:

The DNA sequence encoding the native *C. botulinum* serotype B C fragment gene derived from the Eklund 17B strain can be expressed using the pETHisb vector; the resulting coding region

is listed in SEQ ID NO:43 and the corresponding amino acid sequence is listed in SEQ ID NO:44. The DNA sequence encoding the native *C. botulinum* serotype B C fragment gene derived from the Danish strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:45 and the corresponding amino acid sequence is listed in SEQ ID NO:46. The C ~~frgament~~ fragment region from any strain of *C. botulinum* serotype B can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type B 2017 strain.

The paragraph on page 230, line 12, has been amended as follows:

A number of expression vectors were constructed to facilitate the expression of recombinant BotB protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (lacIq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of [[a]] an optimal expression system for fermentation scaleup.

The paragraph on page 235, line 4, has been amended as follows:

Fermentations were performed to determine if the simultaneous overexpression of folding chaperones (*i.e.*, the Gro operon) and the BotB protein resulted in enhanced solubility of the [[Bot B]] BotB protein. This example involved fermentation of the pHisBotBkan lacIq T7lac/pACYCGro BL21(DE3), pHisBotB kan T7lac/pACYCGro Bl21(DE3) and pHisBotBkan lacIq T7/pACYCGro BL21(DE3) cell lines. Fermentation was carried out as described

in Example 31; 34 μg/ml chloramphenicol was included in the feeder and fermentation cultures.

The paragraph on page 236, line 3, has been amended as follows:

Induction was with 4 gms IPTG at 1 hr post initiation of the glucose feed. The OD_{600} was 33.5 at time of induction, then 44, 51, 58.5 and 69 at 1-4 hrs post induction. Viable colony counts decreased after 2 hrs induction (43, 65, 74, 0 (70), 0 (70) at 0-4 hr induction; bracketed numbers represent microcolonies; dilution 3 utilized 3 μl of dilution 2 cells). Most colonies at induction retained the BotB plasmid (kan ~~resistant) and~~ resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

The paragraph on page 236, line 16, has been amended as follows:

A small scale IDA purification of BotB protein from a 250 ml PEI clarified 15% w/v extract (37.5 gm cell pellet) yielded approximately 12.5 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone (assessed by Coomassie staining of a 10% SDS-PAGE gel). The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that all of the BotB protein produced by the pHisBotB kan T7lac/pACYCGro/Bl21(DE3) cells was soluble; the BotB protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 75 gm cell pellet, this indicated that the yield of soluble affinity purified ~~bot B~~ BotB

protein from this fermentation was 12.5 mg/liter. These results also demonstrated that additional purification steps are necessary to separate the chaperone proteins from the BotB protein.

The paragraph on page 237, line 10, has been amended as follows:

Total and soluble extracts were resolved on a 10% SDS-PAGE gels and Western blotted and his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels (observed by ponceau S staining), and a much higher expression of soluble ~~Bot B~~ BotB protein (compared to expression in the pHisBotB kan T7lac/pACYCGro fermentation) was observed at all timepoints, including uninduced cells (some increase in BotB protein levels were observed after induction).

The paragraph beginning on page 238, line 26, has been amended as follows:

To enhance the solubility of the recombinant BotB protein during scale-up, the protein was co-expressed with folding chaperones (see Ex. 39). During the Ni-IDA purification step, the folding chaperones co-eluted with the BotB protein in 800 mM imidazole; therefore, a second purification step was required to isolate the BotB protein free of folding chaperones. Lane 3 of Figure 35 contains proteins eluted from an IDA column to which a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells had been applied; the proteins were resolved on a 4-15% polyacrylamide pre-cast gradient gel (Bio-Rad, Hercules, CA) run under native conditions and then stained with Coomassie blue. In Figure 35, lanes 1 and 4 contain proteins present in peak 1 and peak 2 from a Sephacryl S-100 column run as described below; lane 2 is blank.

The paragraph on page 239, line 8, has been amended as follows:

As seen in lane 3 of Figure 35, the IDA-purified sample consists primarily of the folding chaperones and the BotB protein. The fact that the chaperones and the ~~Bot B~~ BotB antigen appear as two distinct bands under native conditions suggested they were not complexed together and therefore, it should be possible to separate them, using either a gradient of imidazole concentrations or size exclusion methods.

The paragraph on page 243, line 16, has been amended as follows:

The BotB protein purified by ultrafiltration through the YM 100 membrane was examined for endotoxin using the LAL assay (Associates of Cape Cod) as ~~describe~~ described in Example 24. Two aliqouts of the YM 100-purified BotB preparation were analyzed and were found to contain either 18 to 36 EU/mg recombinant protein or 125 to 250 EU/mg recombinant protein.

The paragraph on page 248, line 7, has been amended as follows:

A number of expression vectors were constructed to facilitate the expression of recombinant BotE protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (lacIq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of [[a]] an optimal expression system for fermentation scaleup. This example

involved a) construction of BotE expression vectors and b) determination of expression levels in small scale cultures.